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PROGRESS TOWARD DEVELOPMENT OF DIAGNOSTIC TESTS FOR GREEN TURTLE FIBROPAPILLOMATOSIS. PART I. MONOCLONAL ANTIBODIES FOR THE MEASUREMENT OF CLASS-SPECIFIC ANTIBODY RESPONSES IN THE GREEN TURTLE, *CHELONIA MYDAS*[*]

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PREFACE

This report was prepared in part as the result of a contract issued to Dr. Paul A. Klein by the Southwest Fisheries Science Center Honolulu Laboratory's Marine Turtle Research Program. The report describes significant progress recently made in research to develop diagnostic tests for fibropapillomatosis in the green turtle, *Chelonia mydas*. This debilitating and often fatal tumor disease of unknown etiology represents a serious threat to green turtle populations in Hawaii, Florida, and certain other sites worldwide. The disease is believed to be increasing in both geographical scope and magnitude.

It is recommended that the reader also refer to Southwest Fisheries Science Center Administrative Report H-94-11C which constitutes Part II of the results of this contracted research by Dr. Klein.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

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Zoologist
December 1994

ABSTRACT

Monoclonal antibodies (Mabs) were developed against the known immunoglobulin classes of the green turtle, *Chelonia mydas*. Plasma protein fractions enriched for 5.7S IgY, 7S IgY, and IgM turtle immunoglobulins were used to immunize Balb/c mice for hybridoma production and for hybridoma screening. Fifteen hybridomas produced Mabs with specificity for turtle immunoglobulins and for affinity purified DNP-specific turtle antibodies. Three Mabs specific for either turtle 5.7S IgY heavy chain (HL814), 7S IgY heavy chain (HL857), or IgM heavy chain (HL846) were purified and used in an ELISA to measure antibody responses in two turtles immunized with DNP-BSA over a ten month period. In both turtles the 7S IgY antibody response developed within 5 weeks of the first inoculation and remained high over the next nine months. The 5.7S IgY antibody response was detected in one turtle at 3-4 months and in the other at 8 months, and reached high levels in both individuals by 10 months. The IgM responses were difficult to interpret. One turtle had pre-inoculation anti-DNP IgM antibody in its plasma and the other developed only a weak, transient response at about 4 months. The class-specific antibody activity in immune turtle plasma could be strongly inhibited by soluble DNP or by rabbit anti-DNP specific antiserum, showing that these antibody responses were directed predominantly to the DNP hapten on the DNP-BSA antigen. Antibody responses to the BSA carrier could not be detected in either turtle over the course of the immunization. Mab HL814, specific for an epitope on the 5.7S green turtle immunoglobulin heavy chain, will be useful for characterizing the molecular relationships of 5.7S, 7S and IgM heavy chains and the role of 5.7S antibody in humoral immunity in this species. All anti-turtle Ig Mabs were screened against the plasma globulins of loggerhead (*Caretta caretta*), olive ridley (*Lepidochelys olivacea*), kemp's ridley (*Lepidochelys kempii*), hawksbill (*Eretmochelys imbricata*), and leatherback (*Dermochelys coriacea*). While the Mabs specific for IgM and 5.7S IgY reacted only with the green turtle, two Mabs specific for light chain reacted with all species except the leatherback, and eight mabs specific for 7S IgY heavy chain reacted with all five species. Thus, these Mabs may be useful for immunodiagnostic applications in these endangered species as well.

KEY WORDS

Immunoglobulin, antibody, monoclonal, turtle.

ABBREVIATIONS

BSA, bovine serum albumin; DNP, dinitrophenol; HAT, hypoxanthine, aminopterin, thymidine; Ig, immunoglobulin; Mabs, monoclonal antibodies; SDS-PAGE, sodium dodecyl sulphate-polyacrylimide gel electrophoresis.

INTRODUCTION

There are 7 extant species of marine turtles all of which are threatened or endangered due to a variety of factors such as over-harvesting, loss of nesting and feeding habitats, marine pollution, and entanglement (National Research Council, 1990). The impact of disease and the relationship of disease susceptibility to environmental factors are poorly understood, due in part to the lack of diagnostic reagents with which to monitor the health status of sea turtle populations. The importance of improved health monitoring capabilities in wildlife conservation is becoming increasingly recognized (Klein, 1993). The urgent need to develop diagnostic tests for green turtles, *Chelonia mydas* stems in part from recent worldwide increases in the prevalence and severity of green turtle fibropapillomatosis and the need to better understand the epizootiology of this disease (Balazs and Pooley, 1991; Jacobson et al., 1989).

The development of standardized serodiagnostic tests for green turtles would be facilitated by the availability of monoclonal antibodies (Mabs) to specific turtle immunoglobulin classes. Monoclonal antibodies are highly specific and uniform reagents with reliable performance characteristics that can be obtained in potentially unlimited quantities. This paper describes the production and validation of a battery of monoclonal antibodies specific for each of the known immunoglobulin classes of the green turtle (Benedict and Pollard, 1972).

MATERIALS AND METHODS

Turtle plasma samples and turtle immunizations

Blood samples were collected into lithium heparin tubes from the dorsal cervical sinus (Owens and Ruiz, 1980) of 4 green turtles housed in a rehabilitation facility in Marathon, Florida. The plasma obtained from these samples was used to prepare immunoglobulins for use as antigen in hybridoma production. Two 14 month old captive-reared green turtles housed at Cayman Turtle Farm, Grand Cayman, British West Indies were immunized by biweekly subcutaneous inoculations with 250 μg 2,4- dinitrophenylated bovine serum albumin (DNP-BSA) (Molecular Probes, Eugene, OR, USA) in Ribi's adjuvant (RIBI ImmunoChem Research, Hamilton, MT, USA) for a total of 6 inoculations, followed by monthly inoculations of the same DNP-BSA dose for another 8 months. Water temperatures at Cayman Turtle Farm ranged from 26.5 to 30°C during this period. A pre-immunization blood sample was collected, followed by biweekly test bleedings between each of the first 6 inoculations, and monthly bleedings before each monthly booster inoculation. These plasma samples were used to assess the ability of Mabs to measure specific turtle anti-DNP responses and for affinity purification of anti-DNP antibodies. In addition, pooled plasma samples from loggerhead (*Caretta caretta*), olive ridley (*Lepidochelys olivacea*), kemp's ridley (*Lepidochelys kempii*), hawksbill (*Eretmochelys imbricata*), and leatherback (*Dermochelys coriacea*) were obtained from various sources for testing cross-species reactivity of the Mabs. All work was conducted under the appropriate state and federal permits and with IACUC approval.

Preparation of turtle immunoglobulins

Several strategies were employed to isolate and purify turtle immunoglobulins for use in mouse immunizations and hybridoma screening. Initially, putative immunoglobulins were identified and isolated according to their physico-chemical properties. Later, additional approaches were taken, as reagents and antigen specific antibodies from specifically immunized turtles became available.

Globulins from a 50 ml sample of plasma from an individual green turtle and from a 100 ml pooled sample from 3 green turtles from Marathon, Florida were precipitated with saturated ammonium sulphate (33% v/v). The precipitate was resuspended in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.02% NaN_3 (PBS/az) and the precipitation repeated. The precipitate was dialyzed into either PBS/az or 0.01 M Tris buffer (pH 8.0) and adjusted to a final protein concentration of 2 mg/ml. One portion (5 ml) of the globulin preparation (33% SAS cut) in Tris buffer was applied to a diethylaminoethyl (DEAE) anion exchange column and eluted in steps with 0.01 M Tris buffer containing either 0.125 M NaCl, 0.25 M NaCl, 0.5 M NaCl, or 1.0 M NaCl. Another portion (18 ml) of the globulin preparation in PBS/az was applied in 6 ml sample amounts to a 2.5 x 100 cm Sephacryl S-300 column in order to separate proteins on the basis of their size. Fractions were eluted with PBS/az at a 30 ml/hr flow rate and collected using a Gilson fraction collector. Selected eluted protein fractions were reduced by boiling for 5 minutes in Laemmli sample buffer (Laemmli, 1970) with 2-mercaptoethanol, and examined by SDS polyacrilamide gel electrophoresis (SDS-PAGE) using a Phastgel apparatus (Pharmacia LKB, Uppsala, Sweden). Fractions containing similar protein composition were pooled and concentrated in centrifuge filter concentrators (Amicon Centriprep^R-10, W.R. Grace & Co, Beverly, MA, USA). Selected DEAE fractions were used to immunize mice and the gel filtration fraction pools were used as antigen in preliminary hyrbridoma screening protocols.

Immunoglobulin purification by anti-light chain affinity column chromatography

An affinity column was prepared using 2 mg monoclonal antibody HL673 which is specific for the immunoglobulin light chain of the desert tortoise (Schumacher et al., 1993). Mab HL673 which had been found to cross-react strongly with putative light chain of the green turtle in ELISA and Western Blots was covalently linked to a hydrazide support gel (Affi-prep^R Hz, Bio-Rad Laboratories, Richmond, CA, USA) following manufacturers instructions. Briefly, 1 ml of purified Mab HL673 (2 mg/ml) was oxidized with 20 μl of sodium periodate stock solution (0.5 M $NaIO_4$) in oxidation buffer (0.02 M sodium acetate, 0.15 M NaCl, pH 5.0) for 45 minutes at room temperature. The oxidation reaction was stopped by addition of 5 μl glycerol. The oxidized antibody was dialyzed into coupling buffer (0.1 M sodium acetate, 1.0 M NaCl, pH 4.5) and incubated overnight with approximately 2 ml of settled hydrazide support beads. The antibody coupled beads were then washed with 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.5 and stored at 4°C. The columns was prepared, conditioned with elution buffer (0.1 M glycine, pH 2.7), washed with PBS/az, and then 1 ml (2mg) of green turtle immunoglobulin rich preparation (33% SAS cut) was applied. After washing the column the bound protein was eluted with 0.1 M

glycine, pH 2.7. Fractions (1 ml) were collected and neutralized with 45 µl of 1.0 M Tris, 0.01% NaN_3). Eluted proteins were concentrated and examined with SDS-PAGE. These proteins were also used to immunize mice for hybridoma production.

Purification of anti-DNP antibodies by affinity column chromatography

Turtle anti-DNP antibodies were purified using affinity chromatography (Wofsy and Burr, 1969; Goetzl and Metzger, 1970). N e-2,4- DNP-lysine (Sigma Chemical Co, St. Louis, MO, USA) (2 mM in 0.1 M $NaHCO_3$, pH 8.3) was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia LKB, Uppsala, Sweden). The DNP-lysine coupled Sepharose was packed into a column 2.5 x 13 cm (37 ml) and washed with 50 ml methanol and then equilibrated in borate buffer (0.015 M $NaBO_3$, 0.15 M NaCl, pH 8.0). The column was further washed with 1% bovine serum albumin (in PBS/az) and 25% acetic acid followed by equilibration in high salt borate buffer (0.015 M $NaBO_3$, 0.5M NaCl, pH 8.0) before use. Pooled plasma from the two DNP-BSA immunized turtles was diluted 1:3 in high salt borate buffer and applied to the column. The column was washed until the OD_{280nm} returned to baseline, and then any bound turtle anti-DNP antibodies were eluted with 5 ml 0.1 M 2,4 DNP-glycine (pH 8.6). The eluted fractions were pooled and concentrated to a 2ml volume and then extensively dialyzed against PBS/az. This solution, containing highly purified turtle anti-DNP antibodies, as judged by ELISA and SDS-PAGE, was used for final screening of newly developed monoclonal antibodies. A small aliquot was dialyzed against 50 mM tris (pH 7.4) for mass spectrophotometry.

Mass spectrometry

Affinity purified turtle anti-DNP antibodies were submitted to the Protein Analysis Core, Interdisciplinary Center for Biotechnology Research, University of Florida for mass spectrometry (Vestec VT 2000, Perseptive Biosystems/Vestec Mass Spectrometry Products, Houston, TX, USA).

Hybridoma production

Mouse immunization protocols

One 6-8 week old female Balb/c mouse was immunized subcutaneously with 6 µg of HL673 affinity purified turtle immunoglobulin in Ribi's adjuvant. Booster immunizations were repeated in two and four weeks. The final booster was 17 µg of antigen intraperitoneally. Fusion was performed 4 days after the last inoculation. Two 6-8 week old female BALB/c mice were immunized with a DEAE fraction (50 µg total protein) containing both 5.7S and 7S green turtle immunoglobulins (see results) in Ribi's adjuvant at several subcutaneous sites. Booster immunizations were performed at 2 weeks, 4 weeks (50 µg antigen per mouse). Immunizations of both mice (100 µg antigen each) were continued at 2 to 4 week intervals for a total of 7 immunizations using a combination of the 5.7S and 7S IgY rich DEAE fraction (25-75 µg) and various IgM-rich preparations derived from DEAE and Sephacryl S300 chromatography runs (45-100 µg). The two mice differed only in the last immunization. One mouse was rested for about 4 weeks before its final booster with both IgY and IgM whereas the second mouse was rested for 10 weeks

before its final booster with turtle IgM alone. Serum anti-turtle titers were checked periodically by ELISA.

Fusions

Monoclonal antibody production followed the standard protocol of the Hybridoma Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida (Liddell and Cryer, 1991; Simrell and Klein, 1979). Three independent fusions (one for each mouse) were carried out. In general, four days following the final booster immunization, mice were euthanized under methoxyflurane anesthesia and their spleens removed. Splenocytes were prepared by mechanical disaggregation, washed, and fused with log phase SP2/0 mouse myeloma cells in a 7:1 ratio using 50% polyethylene glycol 1500 media (Boehringer Mannheim, Germany). After pelleting by centrifugation at 400 x g for 8 minutes, cells were resuspended in fusion media ($DMEA_{(GL)}$, 1 x HAT, 25% SP2/0 Conditioned Media, 20% Horse Serum) (GIBCO, Grand Island, NY, USA) and seeded into 96 well culture plates (Costar, Cambridge, MA, USA). Wells were monitored microscopically for growth of hybridomas.

Screening was begun on growth positive wells 10-14 days post fusion. The supernatants were removed and tested for antibody reactivity against specific antigens (see below). Hybridoma cultures of interest were transferred to 24 well plates and expanded until they could be retested (about 7 days). Hybridoma cultures of interest were safeguarded by cryopreservation in liquid nitrogen. Selected cultures were isotyped using an isotyping kit (Amersham Mouse Monoclonal Antibody Isotyping Kit, Code RPN.29, Amersham, UK) and cloned by limiting dilution.

Monoclonal antibody screening protocols

Hybridoma culture supernatants were screened against each of the three turtle immunoglobulin rich pools derived from the S300 gel filtration column using enzyme linked immunosorbent assays (ELISA). Secondary screening was done by Western Blotting.

ELISA protocol

A standard ELISA protocol was used for screening (Schumacher et al., 1993). Each well of a microtiter plate (Maxisorp F96; NUNC, Kamstrup, Denmark) was coated with 50 μl of antigen at a concentration of 10 μg/ml in PBS/Az and incubated at 4°C overnight. The wells were washed four times with PBS/Az containing 0.05% Tween-20 (PBS-Tween) by an automatic ELISA washer (EAW II; LT-Laboratories, Salzburg, Austria) and then blocked with 300 μl/well of 1% BSA in PBS/Az at room temperature for 60 min or at 4°C overnight. After four more washes, 50 μl of hybridoma culture supernatant was added to individual wells and incubated at room temperature for 60 min. The wells were washed again and 50 μl of a 1:1000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG whole molecule (Sigma Chemical Co, St. Louis, MO, USA) was added to each well. Following incubation at room temperature for 60 min, the plates were washed 4 times with PBS-Tween and 100 μl of p-nitrophenyl phosphate disodium (Sigma Chemical Co, St. Louis, MO, USA) (1 mg/ml prepared in 0.01 M sodium bicarbonate buffer, pH 9.6

containing 2 mM $MgCl_2$) was added to each well and incubated in the dark at room temperature for 90 min. The optical density of each well at a wavelength of 405 nm was measured in an ELISA plate reader (EAR 400 AT; SLT-Laboratories, Salzburg, Austria) at 30, 60, and 90 minutes. Positive and negative controls included on each assay plate consisted of immune mouse serum and hybridoma cell culture medium, respectively. Preliminary screens used selected gel filtration fraction pools (either IgM rich, 5.7S rich, or 7S rich) as antigen (Figure 1). Later ELISA screens used affinity purified turtle anti-DNP antibodies (2 μg/ml) as antigen.

Immunoblotting (Western Blotting)

Immunoblotting (Western Blotting) was performed to help demonstrate the specificity of our monoclonal antibodies for immunoglobulin chains. Immunoblots were prepared following a published basic protocol (Schumacher et al., 1993). Briefly, 100-150 μg of green turtle globulins (33% SAS cut) were separated by SDS-PAGE under reducing conditions, using a precast 10% Tris-Glycine gel (Novex, San Diego, CA, USA) as previously described (Laemmli, 1970). The proteins were then electrophoretically transferred from the gel to a nitrocellulose sheet (Schleicher & Schuell, Keene, NH, USA) using a transfer apparatus (Novex, San Diego, CA, USA). A Tris-glycine buffer (pH 8.3) in 20% methanol was used as transfer buffer. Blotting time was 120 min at 30 volts. Once the transfer was complete, the nitrocellulose was blocked immediately with 5% nonfat dry milk in PBS/Az and incubated at room temperature on a rocker overnight. The membrane was then washed three times (5 min per wash) with PBS-Tween and placed into a trough-manifold (PR 150 Mini Deca Probe; Hoeffer Scientific Instruments, San Francisco, CA, USA). Three hundred μl of hybridoma culture supernatant were loaded per channel and incubated on the nitrocellulose for 90 min at room temperature on a rocker. The nitrocellulose membrane was washed 3 more times and then incubated with 300 μl of a 1:1000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG whole molecule for 90 minutes at room temperature. The membrane was then removed from the manifold, washed 3 times and developed with substrate buffer (0.1 M Tris HCl, 1 mM $MgCl_2$, pH 8.8) containing 44 μl of nitroblue tetrazolium chloride (NBT) and 33 μl of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) per 10 ml of substrate buffer (Immunoselect, GIBCO BRL, Gaithersburg, MD, USA). Immunoblots using biotinylated Mabs followed the same basic procedure except that biotinylated Mabs diluted in 1% BSA-PBS/az to 1 μg/ml replaced hybridoma culture supernatants and strepavidin-alkaline phosphatase (Zymed Laboratories, San Francisco, CA, USA) replaced the alkaline phosphatase conjugated rabbit anti-mouse.

Monoclonal antibody purification and biotinylation

Selected cloned hybridoma lines were injected i.p. into Pristane-primed BALB/c mice and the resulting ascites fluid containing the desired monoclonal antibodies was harvested. Monoclonal antibodies were purified from ascites by passage over a Protein G Sepaharose Fast Flow affinity column (Pharmacia LKB, Uppsala, Sweden) and biotinylated.

Each purified Mab was dialyzed against 0.1 M $NaHCO_3$, pH 8.0 and adjusted to a

final concentration of 1.0 mg/ml (Goding, 1986). Sulphosuccinimidyl-6-(biotinamido) hexanoate (Immuno Pure NHS-LC Biotin; Pierce, Rockford, IL, USA) dissolved in dimethyl sulphoxide at 1.0 mg/ml was added (120 μg biotin per mg of antibody) and the mixture was incubated for 2 hours at room temperature. Following incubation, the Mabs were dialyzed into PBS/az and stored at 4°C.

Cross species reactivity of monoclonal antibodies

Supernatants from hybridomas producing mabs specific for green turtle immunoglobulins were screened by ELISA for reactions with 33% SAS globulin preparations of 5 other sea turtle species. The ELISA followed the general procedure but used each 33% SAS cut at 5 μg/ml coating concentration as antigen.

Verification of monoclonal antibody and turtle antibody specificity

The following experiments were conducted to prove further that developed Mabs were indeed specific for individual turtle immunoglobulin classes and would react with turtle antibodies.

Sandwich ELISA protocol

An antigen capture experiment was designed to test whether the turtle plasma proteins bound by each Mab possessed an immunoglobulin light chain. ELISA plates were coated with 50 μl per well selected purified monoclonal antibody (5 μg/ml). Following incubation with green turtle globulin prep (2 μg/ml), the sandwich was completed with 1 μg/ml biotinylated HL673 (anti-light chain) and detected with strepavidin-alkaline phosphatase.

Detection of immune responses to DNP-BSA

Biotinylated Mabs were used in an ELISA format to measure anti-DNP antibody responses in turtles immunized with DNP-BSA. The general ELISA protocol (described above) was used except that Polysorp plates (Polysorp; NUNC, Kamstrup, Denmark) were coated with 50 μl per well DNP-BSA (1 μg/ml) and blocked with 2.5% casein (pH 7.0). Plasma samples from DNP-BSA immunized turtles were diluted 1:50 in 1% BSA-PBS/az and serial two-fold dilutions were tested. Plates were incubated with class specific biotinylated monoclonal anti-turtle antibody (1 μg/ml in 1%BSA-PBS/az) followed by strepavidin-alkaline phosphatase (Zymed Laboratories, San Francisco, CA, USA).

Competitive inhibition assays

Competitive inhibition ELISA's were used to verify that the turtle plasma proteins, i.e. antibodies, detected by each Mab were DNP-specific. First, plasma samples with peak anti-DNP responses from the 2 immunized turtles were serially diluted and incubated at 4°C overnight with increasing concentrations of soluble hapten (2,4 DNP-glycine pH 7.4 in PBS/az final concentration range: 0-1 mM). These "inhibited" plasma samples were then assayed for residual antibody activity by ELISA as described above. Second, plasma

samples with peak anti-DNP responses were serially diluted and mixed with serial twofold dilutions of rabbit anti-DNP antiserum (Sigma Chemical Co., St. Louis, MO) with specific anti-DNP antibody concentrations ranging from 0 to 7.5 μg/ml. The residual turtle DNP-specific antibody activity was then assayed by ELISA.

RESULTS

Immunoglobulin purification

Turtle globulins eluted from the DEAE column in two peaks corresponding to 0.125 M NaCl and 0.25 M NaCl. Analysis by reducing SDS-PAGE revealed that the 0.125 M NaCl peak contained three major protein components with approximate molecular weights of 23 Kd, 38 Kd, and 65 Kd, consistent with immunoglobulin light chain, 5.7S heavy, and 7S heavy chains respectively (see Ambrosius, 1976). The 0.25 M peak contained a mixture of proteins of various sizes, including a 70 Kd band consistent with IgM heavy chain (data not shown).

Turtle globulins separated on the Sephacryl S-300 column also eluted in two major peaks: an early peak containing putative IgM and a large late peak containing a mixture of 5.7S and 7S IgY (Figure 1A). Fractions were analyzed by reducing SDS-PAGE and those with similar protein composition were pooled and the resulting pools were designated as either IgM-rich, 5.7S-rich, or 7S-rich (Figure 1B). These were used for preliminary ELISA screening of hybridoma supernatants.

A very small amount of turtle immunoglobulin (< 100 μg) was purified from the globulin preparation with an anti-light chain immunoaffinity column. This material contained three major components of approximately 65 Kd, 38 Kd, and 23 Kd, consistent in size with turtle 7S and 5.7S heavy chains and light chain (data not shown).

Turtle anti-DNP antibodies eluted from the DNP-Sepharose column showed 4 predominant bands on reducing SDS-PAGE. These bands had molecular weights of approximately 23, 38, 65, and 70 Kd as expected of the light chain, 5.7S, 7S, and IgM heavy chains respectively (Figure 2A). This protein was used in ELISA and Western Blotting to screen the monoclonal antibody supernatants and purified biotinylated Mabs. Figure 2B shows representative western blot results for these Mabs and HL673 (anti-tortoise light chain) and demonstrates the specificity of each Mab for its target immunoglobulin chain. The control Mab, HL860 (anti-turtle non-immunoglobulin), did not react with the affinity purified turtle anti-DNP antibody preparation.

An aliquot of affinity purified turtle anti-DNP antibodies was examined by mass spectrometry. The mass spectrometer detected two proteins with molecular weights of 120 and 175 Kd corresponding to the expected molecular weights of intact 5.7S and 7S Ig respectively (data not shown). Native IgM could not be detected by mass spectrophotometry because the size limit for the method is 200 Kd.

Production and characterization of monoclonal antibodies

Hybridoma screening by ELISA against various immunoglobulin rich fraction pools and Western Blotting against turtle globulins (33% SAS cut) yielded 20 hybridomas of interest which were retained for further study. The initial selection of these hybridomas was based on the specificity of their Mabs for turtle proteins of the appropriate physical and chemical properties. However these results were not sufficient proof that these Mabs were specific for turtle antibodies. Further screening by ELISA against affinity purified turtle anti-DNP antibodies showed that only 15 were specific for turtle immunoglobulin classes. Table 1 gives the specificities and isotypes of these 15 Mabs. Ten of these Mabs were specific for 7S IgY heavy chain, whereas 2 Mabs each were specific for the immunoglobulin light chain and IgM heavy chain, and only 1 Mab was specific for the 5.7S IgY heavy chain.

Mabs from the 15 hybridomas that reacted positively with affinity purified turtle anti-DNP antibodies were tested by ELISA against serum globulin fractions (33% SAS cuts) from 5 other sea turtle species. Table 1 shows that several monoclonal antibodies reacted with epitopes that are shared broadly among sea turtle species. Nine of the 7s IgY heavy chain specific Mabs cross-reacted with all sea turtle species. Both light chain specific Mabs cross-reacted with all species except the leatherback. The IgM and 5.7s Mabs on the other hand seemed to be specific for green turtle only.

Verification of monoclonal antibody specificity

Three hybridomas were cloned and their monoclonal antibodies purified. These were designated HL814 (anti-5.7S IgYheavy chain), HL846 (anti-IgM heavy chain), and HL857 (anti-7S IgY heavy chain) respectively. These Mabs were used for further validation experiments. We chose not to prepare purified Mabs against turtle immunoglobulin light chain because of the availability of HL673 (anti-desert tortoise light chain).

An experiment was designed to test whether the turtle plasma proteins being bound by each of these Mabs could be identified as immunoglobulin by the criterion of having an immunoglobulin light chain. Figure 3 shows that proteins, selectively captured from turtle plasma SAS cut by Mabs HL814, HL846, or HL857, in turn bound labelled light chain specific Mab HL673, whereas antigen captured by HL860 (Mab specific for an unidentified 33 kd turtle protein present in SAS cut) failed to bind Mab HL673.

Antibody responses to immunization

Figure 4 A-D shows that both HL814 and HL857 could detect rising anti-DNP antibody responses in both experimentally immunized turtles. A rise in 7S IgY anti-DNP activity was detected in both turtles within 5 weeks of beginning immunizations and remained high for the remainder of the experiment. The rise in 5.7S IgY anti-DNP activity took up to 9 months to reach a maximum in both turtles. Results for IgM (HL846) were less clear. One turtle (#3150) appeared to have a high IgM anti-DNP response in the pre-inoculation sample as well as subsequent samples (Figure 4E), whereas the other turtle

(#4624) showed a weak IgM peak at about 13 weeks (Figure 4F). Various modifications of the ELISA protocol such as using high salt (0.5 M NaCl) buffer, failed to reduce the pre-inoculation putative IgM anti-DNP signal. Neither turtle developed detectable antibody titers to BSA after 10 months of immunization with DNP-BSA.

Figure 5 (A-C) shows that the ELISA reactions of immune plasma having peak anti-DNP antibody titers can be inhibited with increasing concentrations of soluble hapten (DNP-glycine). Both the degree of inhibition attained and the shape of the inhibition curves varied between turtles and among antibody classes assayed. Inhibition ranged from 72 to 97% with 1 μM DNP-glycine. Because no anti-BSA antibody responses could be detected over the 10 month period in either of the turtles immunized with DNP-BSA, we were unable to test whether peak anti-BSA responses would be refractory to inhibition by soluble DNP-glycine. Nevertheless, results of this experiment support the conclusion that these monoclonal antibodies (HL814, HL846, and HL857) recognize DNP specific antibodies. The ELISA reactions of immune plasma with peak anti-DNP antibody titers were also strongly inhibited by increasing concentrations of rabbit anti-DNP specific antibodies (data not shown).

DISCUSSION

Sea turtles have 3 major classes of immunoglobulins: a 17 S IgM, a 7S IgY, and a 5.7S IgY (Benedict and Pollard, 1972). IgM is believed to be produced transiently early in an immune response, as in mammals (Benedict and Pollard, 1977; Chartrand et al., 1971). In reptiles, IgM may be the primary immunoglobulin that is secreted onto mucosal surfaces (Portis and Coe, 1975). 7S IgY is believed to function as a serum antibody like mammalian IgG. The role of 5.7S IgY is unclear, but evidence suggests that it is a chronic immune response globulin and that it is maternally transferred to egg yolk (Benedict and Pollard, 1972; Benedict and Pollard, 1977; Chartrand et al., 1971).

The results presented here show that we have produced monoclonal antibodies with specificity for the light chain and each of the three heavy chain classes of the green turtle. The purification and screening strategies used were dictated in part by the limited availability of turtles and antigen specific plasma. Initially we did not have access to plasma from specifically immunized green turtles so the early rounds of immunoglobulin purification and hybridoma screening relied on identification of plasma proteins with the physico-chemical properties (solubility, size, and charge) consistent with earlier reports on turtle immunoglobulins (Benedict and Pollard, 1972; Benedict and Pollard, 1977; Leslie and Clem, 1972). Previous structural studies of turtle antibodies (several species) indicate that turtle light chains are approximately 22.5 kD and turtle 5.7S IgY, 7S IgY, and IgM heavy chains are 35-38, 63-68 kD, and 70 kD respectively (Ambrosius, 1976; Benedict and Pollard, 1977; Leslie and Clem, 1972; Chartrand et al., 1971). Preliminary screening of fusions yielded a collection of 20 hybridomas that bound to turtle plasma proteins with the appropriate physico-chemical properties. However, additional screenings against antigen specific turtle antibodies (affinity purified turtle anti-DNP antibodies) revealed that only 15 of these Mabs could be classified as immunoglobulin specific. The turtle proteins recognized by the other 5 Mabs had similar physico-chemical properties but could not be

shown to bind antigen.

Additional experiments were conducted to prove further that the selected cloned hybridomas produced monoclonal antibodies that were specific for bona fide turtle antibodies. A sandwich ELISA, using an anti-light chain Mab demonstrated that the turtle plasma proteins recognized by each of the heavy chain specific Mabs possessed a light chain, thereby confirming the specificity of these Mabs for turtle immunoglobulins. Mabs specific for turtle immunoglobulin classes should be able to detect an increasing antibody titer in response to immunization with specific antigen. The monoclonals HL814 and HL857 (5.7S IgY heavy and 7S IgY heavy chain specific, respectively) were able to measure an increasing anti-DNP response in two chronically immunized turtles. The 7S anti-DNP response rose rapidly within 5 weeks whereas the 5.7S response required months to develop. Qualitatively, this pattern is consistent with previously published descriptions of the ontogeny of the immune response in green turtles (Benedict and Pollard, 1977).

The IgM heavy chain specific Mab HL846 demonstrated a weak transient response in one animal but not in the other. The IgM peak occurred approximately 2 months after the 7S response developed, which was inconsistent with our expectations. Other workers have had difficulty in consistently demonstrating the classical mammalian type IgM response in green turtles (Benedict and Pollard, 1977), although it has been demonstrated in other turtle species (Chartrand et al., 1971). The type of antigen, adjuvant, dosage, dosing frequency, and testing frequency may all influence whether an IgM response occurs and is detected. Previous exposure to antigen will also effect the development of an IgM response. The second turtle appeared to have a high anti-DNP IgM titer in the pre-inoculation plasma sample and in all subsequent samples. The occurrence of natural antibodies to DNP and weak DNP binding by non-immunoglobulin plasma proteins has been reported previously in the green turtle (Benedict and Pollard, 1972). IgM titers are notoriously difficult to measure in an ELISA format (Betts Carpenter, 1992; Kuen et al., 1993). Problems can include antibody aggregate formation, nonspecific binding of IgM to ELISA plates, and autoimmune anti-Ig IgM (RF factors). We were able to minimize nonspecific IgM binding by using high salt buffers (1% BSA in PBS adjusted to 0.5 M NaCl) and using Polysorp ELISA plates. Despite these efforts, the anti-DNP IgM activity of the pre-inoculation sample remained high. The 2 green turtles immunized repeatedly with 250 μg doses of DNP-BSA did not develop detectable anti-BSA titers in this study. The results may be explained by the antigen dose that we used, because a previous study (Benedict and Pollard, 1972) that demonstrated anti-BSA titer development used pure BSA at much higher doses (18-80 mg).

The competitive inhibition experiments were designed to demonstrate that the putative anti-DNP responses detected in the immunization experiment were due to increasing plasma levels of anti-DNP specific antibodies. Incubation of high anti-DNP titer plasma with increasing concentrations of soluble DNP antigen prior to incubating the plasma with bound antigen inhibited the ELISA reaction and clearly demonstrated that the turtle antibody responses being measured were predominantly and specifically directed against the DNP hapten on the DNP-BSA antigen. Similarly, increasing concentrations of rabbit anti-DNP specific antibodies competitively inhibited the ELISA reactions of high anti-

DNP titer turtle plasma.

A surprising result of this study was the isolation of a monoclonal antibody (HL814) that was specific for 5.7S IgY heavy chain. Previous work with 5.7S and 7S immunoglobulins of turtles using polyclonal antisera revealed a close antigenic relationship between these two classes (Benedict and Pollard, 1972). The 5.7S heavy chain is believed to be a fragment of the 7S molecule. Molecular analysis of the sequences of 5.7S and 7S heavy chains of ducks, *Anas platyrhynchos*, indicate that, except for the extreme carboxyl terminus of the 5.7S molecule, which is unique, the 5.7S constant region sequences (CH1 and CH2) are virtually identical to its 7S counterpart (Magor et al., 1992). The duck 5.7S molecule is a truncated version of the 7S molecule (lacking 2 terminal exons), most likely derived by differential mRNA splicing. If the relationship between green turtle 5.7S and 7S heavy chains is the same as for the duck, then it should be possible to develop Mabs that recognize epitopes that are common to both 5.7S and 7S and Mabs that are specific for epitopes unique to 7S. Mabs specific for a unique 5.7S epitope should be relatively rare and should recognize the unique terminal portion of the molecule. Our data support the fact that this Mab is 5.7S specific. Mab HL814 detects increasing antigen-specific antibody titers in immunized turtles and binds to a 35 Kd protein that forms a 120 Kd native structure possessing an immunoglobulin light chain. A partial amino acid sequence for this protein is needed to begin studying the homologies of this protein. Mab HL814 is a key reagent for affinity purification of green turtle 5.7S immunoglobulin and the subsequent cloning and sequencing of the 5.7S cDNA for comparative studies with other vertebrate immunoglobulins.

The primary objective for developing these Mabs was to design practical serodiagnostic tests for monitoring populations of endangered green turtles for exposure to pathogens and for studying humoral immune competence in this species. However, the time and effort required to develop and validate these monoclonal antibody reagents warranted a preliminary assessment of their applicability for other threatened sea turtle species. Preliminary screening indicates that our IgM and 5.7S specific Mabs are green turtle species specific but that the 7S IgY specific and light chain Mabs cross react with other sea turtle species. Thus these Mabs may be used to explore humoral immune function and conduct seroepizootiologic studies in all of these endangered species.

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REFERENCES

Ambrosius, H., 1976. Immunoglobulins and antibody production in reptiles. In: J.J. Marchalonis (Editor), Comparative Immunology, Blackwell Scientific, Oxford, pp. 298-334.

Balazs, G. H. and Pooley, S. G., 1991. Research plan for marine turtle fibropapilloma. NOAA Technical Memorandum NMFS. NOAA-TM-NMFS-SWFSC-156, 1991.

Benedict, A. A. and Pollard, L. W., 1972. Three classes of immunoglobulins found in the sea turtle, *Chelonia mydas*. Folia Microbiologica, 17:75-78.

Benedict, A. A. and Pollard, L. W., 1977. The ontogeny and structure of sea turtle immunoglobulins. In: J. B. Solomon and J. D. Horton (Editors), Developmental Immunology. Elsevier Press, Amsterdam, pp. 315-323.

Betts Carpenter, A. ,1992. Enzyme-linked immunoassays. In: N.R. Rose, E.C. deMacario, J. L. Fahey, H. Friedman and G. M. Penn (Editors), Manual of clinical laboratory immunology 4th ed., American Society for Microbiology, Washington DC, pp. 2-9.

Chartrand, S. L., Litman, G. W., LaPointe, N., Good, R. A., Frommel, D., 1971. The evolution of the immune response. XII. The immunoglobulins of the turtle. Molecular requirements for biologic activity of 5.7S immunoglobulins. J. Immun., 107:1-11.

Goding, J. W., 1986. Monoclonal antibodies: principles and practice. Academic Press, London, pp.263-265.

Goetzl, E. J. and Metzger, H., 1970. Affinity labeling of a mouse myeloma protein which binds nitrophenyl ligands. Kinetics of labelling and isolation of a labeled peptide. Biochem., 9:1267-1278.

Jacobson, E. R., Mansell, J. L., Sundberg, J. P., Hajjar, L., Reichmann, M. E., Ehrhart, L. M., Walsh, M., Murru, F., 1989. Cutaneous fibropapillomas of green turtles, *Chelonia mydas*. J. Comp. Pathol., 101:39-52.

Klein, P. A., 1993. Immunology and biotechnology for the study and control of infectious diseases in wildlife populations. J. Zoo. Wildl. Med., 24:346-351.

Kuen, L. S., Ming, C. H., Fan, Y. S., 1993. Background noise in ELISA procedures: influence of the pH of the coating buffer and correlations with serum IgM concentration. J. Immun. Meth., 163:277-278.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

Leslie, G. A. and Clem, L. W., 1972. Phylogeny of immunoglobulin structure and function. VI. 17S, 7.5S, and 5.7S anti-DNP of the turtle, *Pseudamys scripta*. J. Immun., 108:1656-1664.

Liddell, J. E. and Cryer, A., 1991. A Practical Guide to Monoclonal Antibodies. John Wiley & Sons Ltd, Chichester, England.

Magor, K. E., Warr G. W., Middleton, D., Wilson, M. R., Higgins, D. A., 1992. Structural relationship between the two IgY of the duck, *Anas platyrhynchos*: molecular genetic evidence. J. Immun., 149:2627-2633.

National Research Council, 1990. Decline of the sea turtles: causes and prevention. National Academy Press, Washington, DC.

Owens, D. W. and Ruiz, G. J., 1980. New methods of obtaining blood and cerebrospinal fluid from marine turtles. Herpetologica, 36:17-20.

Portis, J. L. and Coe, J. E., 1975. IgM the secretory immunoglobulin of reptiles and amphibians. Nature, 258:547-548.

Schumacher, I. M., Brown, M. B., Jacobson, E. R., Collins, B. R., Klein, P. A., 1993. Detection of antibodies to a pathogenic mycoplasma in desert tortoise (*Gopherus agassizii*) with upper respiratory tract disease. J. Clin. Micro., 31:1454-1460.

Simrell, C. R. and Klein, P. A., 1979. Antibody responses of tumor-bearing mice to their own tumors captured and perpetuated as hybridomas. J. Immun., 123: 2386-2394.

Wofsy, L. and Burr, B., 1969. The use of affinity chromatography for the specific purification of antibodies and antigens. J. Immun., 103:380-382.

Table 1. List of monoclonal antibodies specific for green turtle immunoglobulins.

Hybridoma	Clone	Mab Isotype	Specificity for Turtle Ig*	Cross Species Reactivity
92.137.3-1H9	HL814	IgG_1	5.7S	0
92.137.3-3F6	-	IgG_{2A}	7S	0
92.137.10-3C6	HL857	IgG_{2A}	7S	Cc,Dc,Ei,Lk,Lo**
92.137.10-3E11	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-3C3	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-3G11	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-3B5	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-3E10	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-3B11	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-4C3	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.10-1G10	-	IgG	7S	Cc,Dc,Ei,Lk,Lo
92.137.15-4G11	-	IgG	IgM	0
92.137.15-7A9	HL846	IgG_1	IgM	0
92.137.15-2C12	-	IgG	Light Chain	Cc,Ei,Lk,Lo
92.137.15-2C11	-	IgG	Light Chain	Cc,Ei,Lk,Lo

* Confirmed by Western Blot.

** Cc = *Caretta caretta*, Dc = *Dermochelys coriacea*, Ei = *Eretmochelys imbricata*, Lk = *Lepidochelys kempi*, Lo = *L. olivacea*.

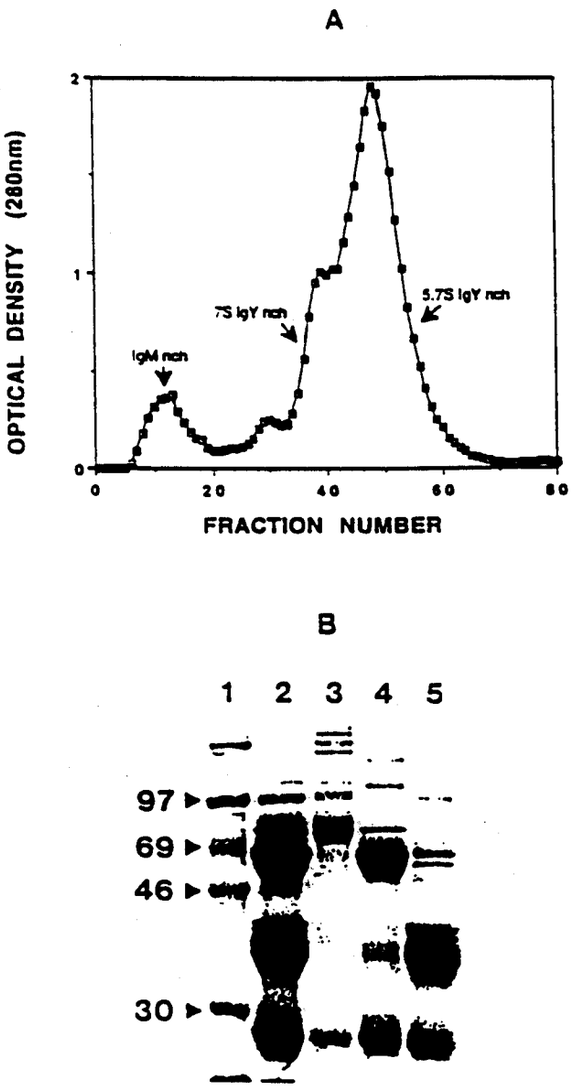

Figure 1. Fractionation of green turtle immunoglobulins by gel filtration chromatography. Turtle globulins (33% SAS cut) were applied to a Sephacryl S-300 column and eluted with PBS/az. Fractions with similar protein composition by SDS-PAGE analysis were pooled. Three fraction pools were produced: IgM rich (fractions 8-18), 7S rich (fractions 34-40), and 5.7S rich (fractions 50-62). (A) Elution profile of turtle globulins fractionated on S-300 column. Protein content of each fraction was estimated by spectrophotometry (OD280nm). (B) Reducing SDS-PAGE (10% Tris-glycine) of green turtle immunoglobulin rich fraction pools, stained with Coomassie Blue. Lane 1 - molecular weight markers (kD); lane 2 - 33% SAS cut; lane 3 - IgM rich pool; lane 4 - 7S IgY rich pool; lane 5 - 5.7S IgY rich pool.

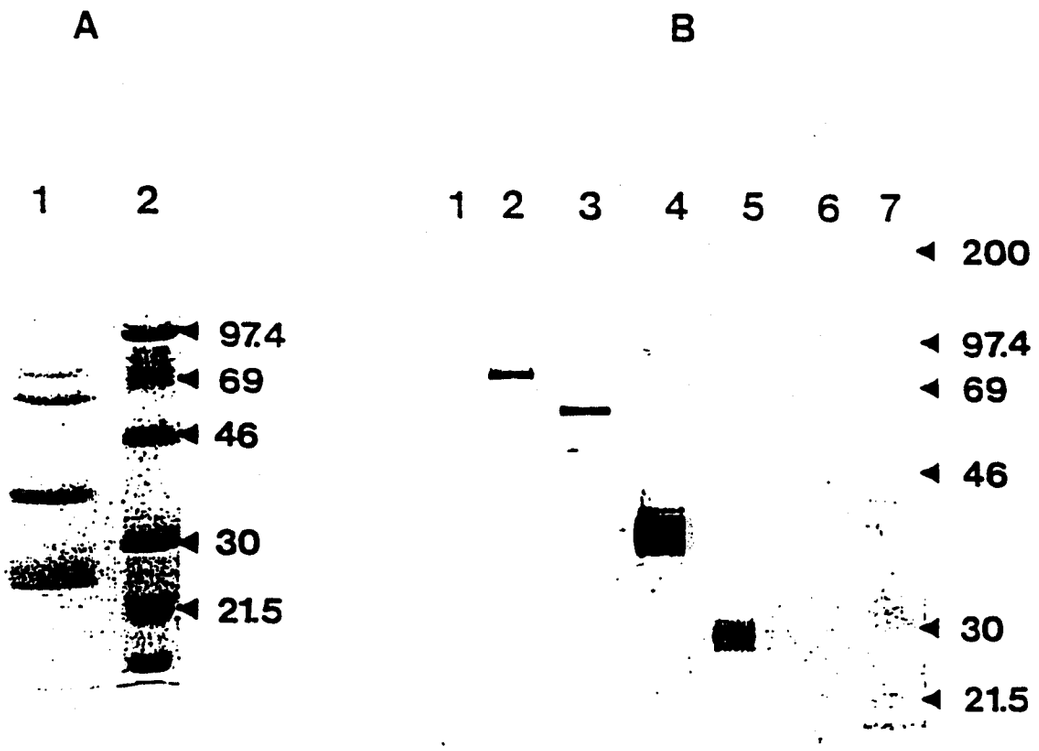

Figure 2. Affinity purified turtle anti-DNP antibody chains. (A) Coomassie Blue stained 12% Tris-glycine reducing gel showing turtle anti-DNP antibodies eluted from a DNP-Sepharose affinity column with 0.1M DNP-glycine. Lane 1 - turtle anti-DNP antibodies; lane 2 - molecular weight markers. (B) Immunoblot of selected monoclonal antibodies on affinity purified turtle anti-DNP antibodies. Each lane was incubated with a different biotinylated monoclonal antibody: either HL860 anti-turtle non-immunoglobulin plasma protein (lane 1), HL846 anti IgM (lane 2), HL857 anti - 7S IgY heavy chain (lane 3), HL814 anti - 5.7S IgY heavy chain (lane 4), or HL673 anti - tortoise IgY light chain (lane 5). 1% BSA was placed in lane 6 as a control. Molecular weight markers were run in lane 7.

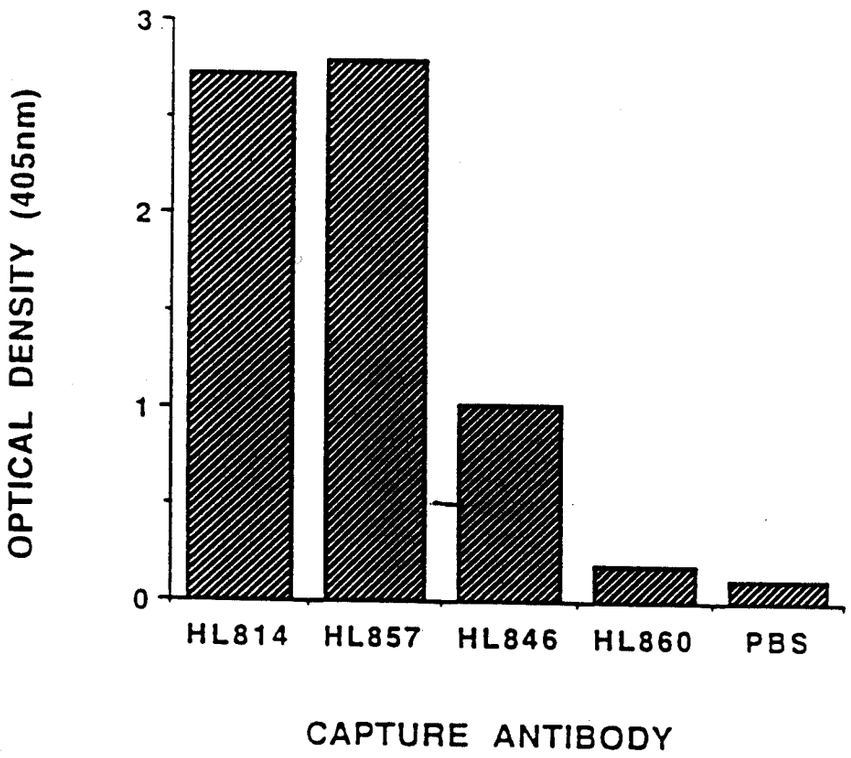

Figure 3. Sandwich ELISA demonstrating that putative heavy chain specific Mabs bind plasma proteins with immunoglobulin light chains. ELISA plates were coated with 5 μg/ml of each purified Mab. HL814 is anti 5.7S heavy chain, HL857 is anti 7S heavy chain, HL846 is anti IgM heavy chain, and HL860 is anti non-immunoglobulin turtle plasma protein. Following incubation with 2 μg/ml turtle globulins (33% SAS cut), plates were washed and incubated with 1 μg/ml biotinylated HL673 (anti-tortoise light chain) to complete the sandwich. Binding of HL673 was detected with strepavidin alkaline phosphatase. Data presented are ELISA reactivities (OD405nm) after 90 minutes incubation with substrate.

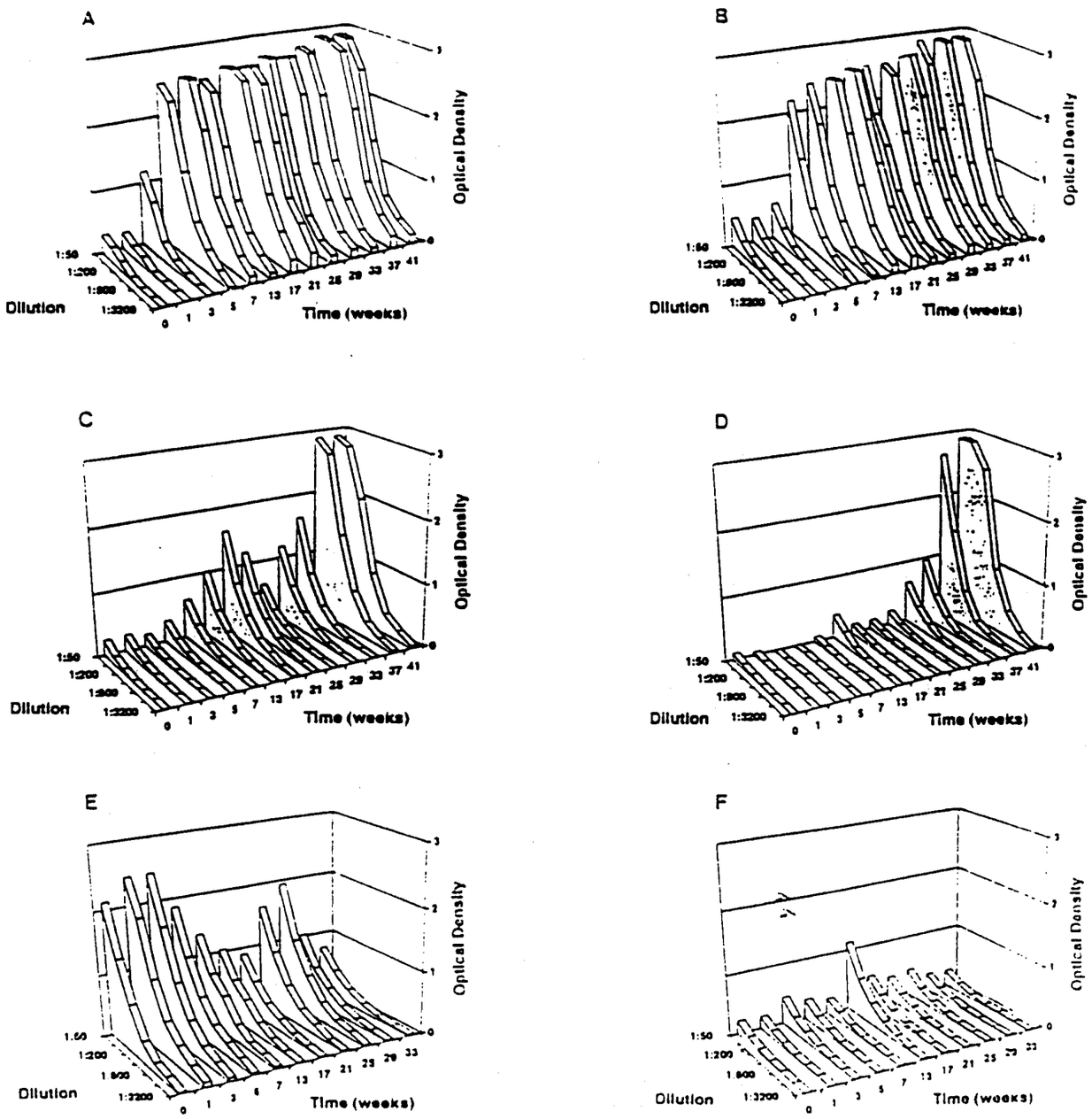

Figure 4. Development of antibody responses to DNP with time in chronically immunized turtles. Plasma samples, collected periodically from 2 turtles during a prolonged immunization schedule (10 months), were serially diluted and tested by ELISA for anti-DNP activity using various biotinylated Mabs. The rise in OD405nm with time is indicative of a rising DNP specific antibody titer. A and B are 7S responses, C and D are 5.7S responses, E and F are IgM responses detected by biotinylated HL857, HI814, and HL846 respectively. Panels A, C, and E show the responses of turtle #3150 and B, D, and F show those of turtle #4624.

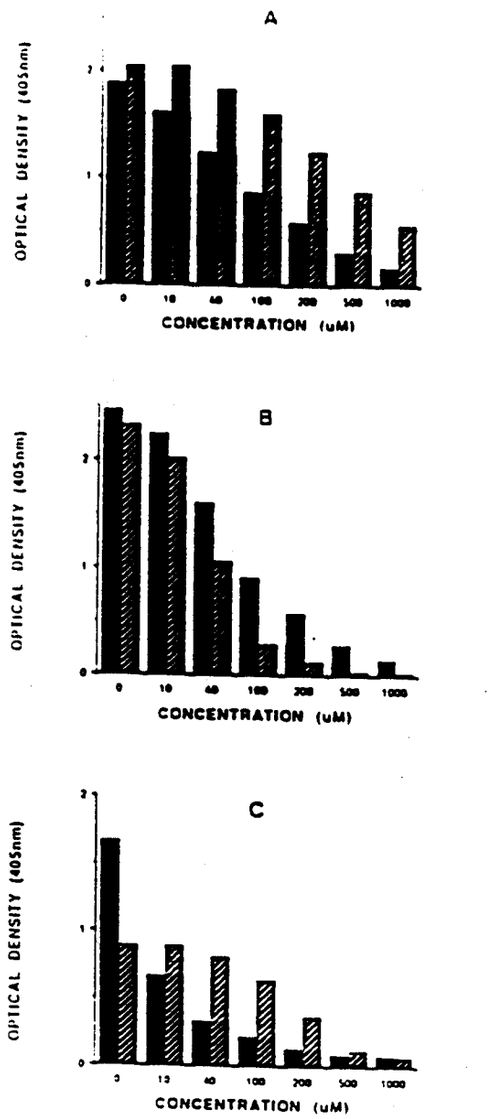

Figure 5. Inhibition of turtle anti-DNP antibody activity by soluble antigen. Serially diluted plasma samples with peak 5.7S, 7S, or IgM anti-DNP antibody activity were incubated overnight with various final concentrations of 2,4 DNP-glycine (0-1 mM final concentration). Samples were then tested by ELISA for binding to DNP-BSA coated plates. OD405nm taken at 60 minutes were plotted against DNP-glycine concentration. The plasma samples used were: week 41 for 5.7S and 7S (both turtles), week 1 for #3150 IgM, and week 13 for #4624 IgM (see Figure 4). The plasma dilutions that are plotted are: 1:400 for 5.7S IgY (HL814) and 7S IgY (HL857) and 1:50 for IgM (HL846). Solid bars represent turtle #3150; crosshatched bars represent turtle #4624.